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A "*Twist box*" Code of p53 Inactivation: *Twist box*:p53 Interaction Promotes p53 Degradation

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SUMMARY

Twist proteins have been shown to contribute to cancer development and progression by impinging on different regulatory pathways, but their mechanism of action is poorly defined. By investigating the role of Twist in sarcomas, we found that Twist1 acts as a mechanism alternative to *TP53* mutation and MDM2 overexpression to inactivate p53 in mesenchymal tumors. We provide evidence that Twist1 binds p53 C terminus through the *Twist box*. This interaction hinders key posttranslational modifications of p53 and facilitates its MDM2-mediated degradation. Our study suggests the existence of a *Twist box* code of p53 inactivation and provides the proof of principle that targeting the *Twist box*:p53 interaction might offer additional avenues for cancer treatment.

INTRODUCTION

Twist1 and Twist2 (collectively hereafter referred as "Twist") are closely related members of a family of bHLH transcription factors involved in gastrulation and mesoderm specification. Typically, Twist proteins regulate the expression of target genes by binding, as homo- or heterodimers, to E-box-containing promoters. Consistent with the role in tissue specification, the expression of *Twist1* in mouse embryo follows mesoderm induction and becomes negligible in adult mesenchymal tissues, except a population of quiescent mesodermal stem cells. *Twist2* is also involved in mesoderm development, but its activation occurs later than *Twist1* and is essentially restricted to the dermis (Barnes and Firulli, 2009; Castanon and Baylies, 2002; Qin et al., 2012; Tukel et al., 2010).

Twist proteins were first associated with cancer on the basis of their ability to promote the bypass of cellular safeguard programs. Both genes were isolated through a genetic screen for cDNAs capable of overriding Myc-induced apoptosis, and Twist1 was found to be overexpressed in rhabdomyosarcomas, where it was suggested to support oncogenic transformation and to inhibit myogenic differentiation (Maestro et al., 1999). Subsequently, de novo Twist1 activation was reported in several types of cancer including neuroblastomas (Valsesia-Wittmann et al., 2004) and carcinomas, where it was shown to contribute to metastatic progression through the induction of epithelial-mesenchymal transition (EMT) (Karreth and Tuveson, 2004; Yang et al., 2004). A role for Twist proteins in stemness has also recently emerged (Cakouros et al., 2010; Vesuna et al., 2009).

Significance

Although sarcomas are relatively rare tumors, their aggressive behavior, resistance to therapies, and often early-age onset make them one of the most challenging types of cancer. Intriguingly, despite clear evidence of attenuation of the p53 response, a large fraction of sarcomas retain wild-type *TP53*, indicating that mechanisms different from mutations account for p53 inactivation in these tumors. Here, we provide evidence that Twist1-induced destabilization of p53 represents an important strategy of attenuation of the p53 response in sarcomas. We show that Twist1 accumulates mostly in tumors that retain wild-type *TP53*. Moreover, we show that, by establishing direct interaction, Twist1 hinders key phosphorylations of p53 and facilitates its degradation. Thus, targeting the Twist1:p53 interaction might offer additional avenues for cancer treatment.

The different consequences of constitutive Twist expression suggest that these transcription factors may contribute to tumorigenesis and neoplastic progression through different routes. In particular, Twist1 has been demonstrated to bind the E-cadherin promoter to suppress its transcription, thus facilitating EMT and metastatic spreading of epithelial tumors (Karreth and Tuveson, 2004; Yang et al., 2004). Twist proteins have also been shown to suppress the transcription of p19ARF, thus attenuating oncogene-induced p53 response, and p16INK4a, thus allowing cancer cells to escape Rb-mediated cell cycle control (Ansieau et al., 2008; Feng et al., 2009; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Li et al., 2009; Maestro et al., 1999; Shiota et al., 2008; Stasinopoulos et al., 2005; Valsesia-Wittmann et al., 2004; Vichalkovski et al., 2010). Moreover, Twist mediates mesenchymal stem cell self-renewal, and Twist1-induced EMT requires BMI1, thus linking EMT and stemness (Isenmann et al., 2009; Yang et al., 2010). Finally, it has been proposed that EMT and bypass of safeguard programs might represent two sides of the same coin (Ansieau et al., 2008). Overall, the emerging picture is that Twist proteins play important roles in cancer, but the fact that they intersect multiple different pathways makes it hard to dissect the mechanisms of action as EMT/metastasis factors and as primary oncogenic drivers.

Sarcomas represent a heterogeneous group of mesenchymal tumors that account for about 5% of adult and 10% of pediatric neoplasias. Sarcomas include over 60 histopathological categories and are broadly classified into two cytogenetic groups, complex and simple karyotype, and these latter are often characterized by reciprocal translocations or targeted amplifications (Borden et al., 2003; Fletcher et al., 2002; Helman and Meltzer, 2003). A large fraction of localized sarcomas, especially the simple karyotype ones, retain wild-type *TP53* but their p53 response is attenuated. Thus, other, still elusive, mechanisms are likely responsible for p53 inactivation in these tumors.

We reasoned that, being mesenchymal in nature, sarcomas offer the opportunity to discern the functions of Twist related to the induction of EMT, typically occurring in carcinomas, from those more specifically related to the interference with tumor-suppressive pathways. Thus, in the attempt to provide a better understanding on how Twist contribute to tumorigenesis, we sought to investigate the role of Twist in antagonizing p53, focusing on sarcomas as a tumor model.

RESULTS

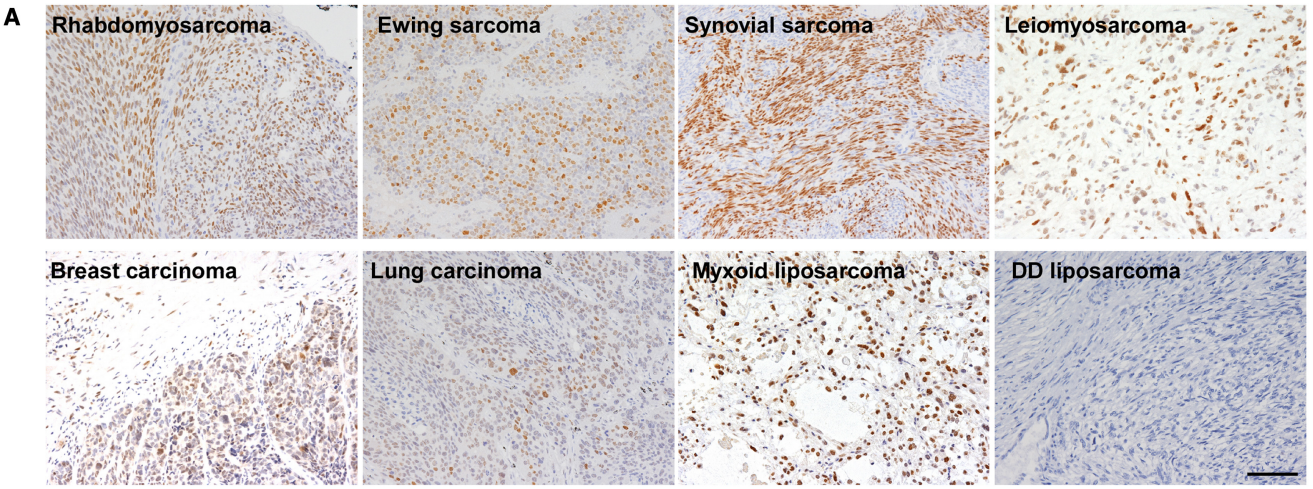
Twist1 Is Overexpressed and Undergoes Copy-Number Gain in Sarcomas

To assess the oncogenic role of Twist in the context of mesenchymal tumors, 146 sarcomas and adjacent normal tissues were investigated by immunohistochemistry (IHC). With the exception of dermal fibroblasts, where scattered nuclear reactivity was observed, normal adult mesenchymal tissues were essentially negative for Twist1. In contrast, a strong and diffuse nuclear accumulation of Twist1 was observed in over 60% of soft-tissue sarcomas of different subtypes (Figure 1A; see Table S1 available online). Overexpression of Twist2 was uncommon in sarcomas (6/84 cases) and sarcoma cell lines (Figure S1A). Fluorescence in situ hybridization (FISH) analyses with a probe encompassing the *TWIST1* locus revealed that, in 9 of 19 (47%) Twist1 IHC$^+$ frozen samples, Twist1 accumulation was associated with *TWIST1* copy-number gain (Figure 1B; Table S2).

In light of the role of Twist1 in EMT, we then compared Twist1 expression pattern in sarcomas and carcinomas. In contrast to the widespread and robust accumulation detected in sarcomas, a weak-moderate nuclear expression of Twist1 was observed in 10%–30% of breast, colorectal, prostate, and lung carcinomas, often confined to the invasion front (Figure 1A; Table S3).

Twist1 Overexpression Serves as an Alternative Mechanism of p53 Inactivation during Sarcomagenesis

Because mutations of *TP53* are rare in sarcomas and Twist1 has been suggested to attenuate the p53 pathway (Ansieau et al., 2008; Feng et al., 2009; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Li et al., 2009; Maestro et al., 1999; Shiota et al., 2008; Stasinopoulos et al., 2005; Valsesia-Wittmann et al., 2004; Vichalkovski et al., 2010), we asked whether the overexpression of Twist1 could account for p53 inactivation in sarcomas retaining wild-type *TP53*. We first focused on leiomyosarcomas (LMS), a sarcoma subtype that has one of the highest frequencies of *TP53* mutations (Dei Tos et al., 1996; Hall et al., 1997). In particular, we analyzed localized/nonmetastatic tumors because sarcomas may acquire p53 mutations that contribute to tumor aggressiveness during progression (Cordon-Cardo et al., 1994). Among the 35 LMS analyzed, overexpression (>25% positive cells) of p53, Twist1, Twist2, and MDM2 were found in 14, 15, three, and four cases, respectively (Table S4). *TP53* missense mutations were found in eight cases (Figure 1C), all displaying strong nuclear accumulation of p53; no mutation was detected in samples that were negative or with focal/patchy p53 immunostaining. Fourteen of 15 Twist1$^+$ cases retained wild-type *TP53*, the only exception being a case that was also positive for MDM2. Thus, although not reaching the conventional 5% level of statistical significance, probably in part because of the small sample size of these clinically rare tumors, the clustering of Twist1-positivity among p53 wild-type LMS suggests that overexpression of Twist1 may serve as a mechanism alternative to *TP53* mutations to inactivate the p53 response in these tumors. To corroborate the role of Twist1 in inhibiting p53, we then focused on liposarcomas (LS), which include well-differentiated (WD) and dedifferentiated (DD) LS, and myxoid/round cell (myxoid) LS. These LS display a simple karyotype and often retain the wild-type *TP53*. WD and the more aggressive DD LS, which are considered the same entity at different malignant stages, typically carry the amplification of the chromosome region harboring the *MDM2* locus (Fletcher et al., 2002), and their p53 is inactivated as a result of enhanced MDM2-mediated degradation. In contrast, the mechanism of inactivation of p53 in myxoid LS, which are negative for MDM2, remains unclear (Coindre et al., 2010; Mentzel and Fletcher, 1995). In a series of 24 LS, all molecularly confirmed to be *TP53* wild-type, we observed an inverse correlation between Twist1 and MDM2 overexpression: Twist1 was robustly overexpressed in 13 of 14 MDM2$^-$ myxoid LS, whereas only 2 of 10 MDM2$^+$ WD and DD LS expressed Twist1 ($p = 0.00049$) (Figures 1A and 1C). Thus, Twist1 and MDM2 appear to be essentially mutually exclusive in LS, supporting the notion that Twist1 may inactivate p53 in mesenchymal tumors.



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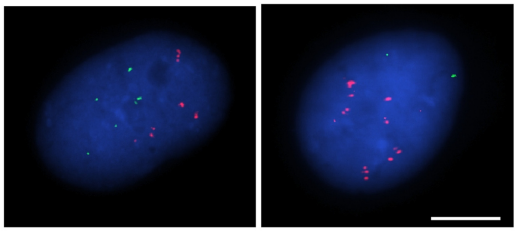


C

		Twist1$^+$	Twist1$^-$	Total	p
LMS	p53 mutated	1	7	8	
	p53 wild-type	14	13	27	
	Total	15	20	35	0.10090
LS	MDM2$^+$ (WD, DD LS)	2	8	10	
	MDM2$^-$ (Myxoid LS)	13	1	14	
	Total	15	9	24	0.00049

D

BJ cells	Oncogene combination	Nr tumors/ Nr injections
RE-vector	Ras + E1A + vector	0/10
RE-MDM2	Ras + E1A + MDM2	9/10
RE-shp53	Ras + E1A + shp53	3/4
RE-dnp53	Ras + E1A + dnp53	7/8
RE-Twist1	Ras + E1A + Twist1	5/8
RE-Twist2	Ras + E1A + Twist2	6/8

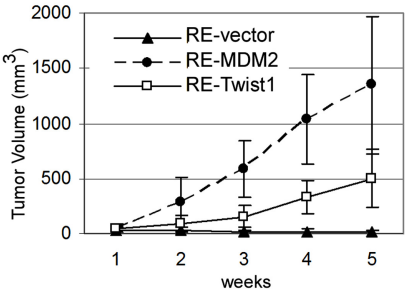


Figure 1. Twist1 Is Overexpressed in Human Sarcomas and Supports the Oncogenic Transformation of Primary Mesenchymal Cells

(A) Immunostaining for Twist1 in sarcomas and in carcinomas. Scale bar: 100 μm.

(B) FISH analysis of *TWIST1* in sarcomas overexpressing Twist1. *TWIST1* probe (RP11-960P19) is in red; chromosome 7 centromeric probe (Alpha-Satellite 7) in green. An example of copy-number gain involving the whole chromosome 7 (left panel) and of selective amplification of the *TWIST1* locus (right panel) are shown. Scale bar: 10 μm.

(C) Expression of Twist1 in LMS according to *TP53* mutation status (upper) and in LS according to MDM2 expression (lower). The p value was calculated according to the Fisher's exact test.

(D) Twist1 and Twist2 induce tumorigenic conversion of RE-BJ cells. The table indicates the number of tumors generated by each oncogenic combination in a set of injections. MDM2, a dominant-negative *TP53* allele (dnp53, R175H), and a shRNA targeting p53 (shp53) were used as positive controls. Representative images of RE-vector and RE-Twist1 xenografts are shown in the middle panel. Tumor size was measured weekly. The right panel shows the kinetics (average volume of tumors, ± SD) of representative tumors.

See also Figure S1 and Tables S1, S2, S3, and S4.

To functionally validate the role of Twist1 as an antagonist of p53 during sarcomagenesis, we then probed its ability to complement the transformation of human primary fibroblasts. We showed previously that BJ human fibroblasts upon expression of HRasV12 and E1A (RE-BJ) gain a transformed phenotype in culture but display negligible tumorigenic activity in xenograft assays. RE-BJ cells become tumorigenic following inactivation of the p53 pathway (Di Micco et al., 2006; Seger et al., 2002). We found that Twist-transduced RE-BJ also generated tumors when injected into immunocompromised mice, supporting that Twist proteins may actually sustain oncogene-induced transformation of cells of mesenchymal origin at least in part by antagonizing p53 (Figures 1D, S1C, and S1D).

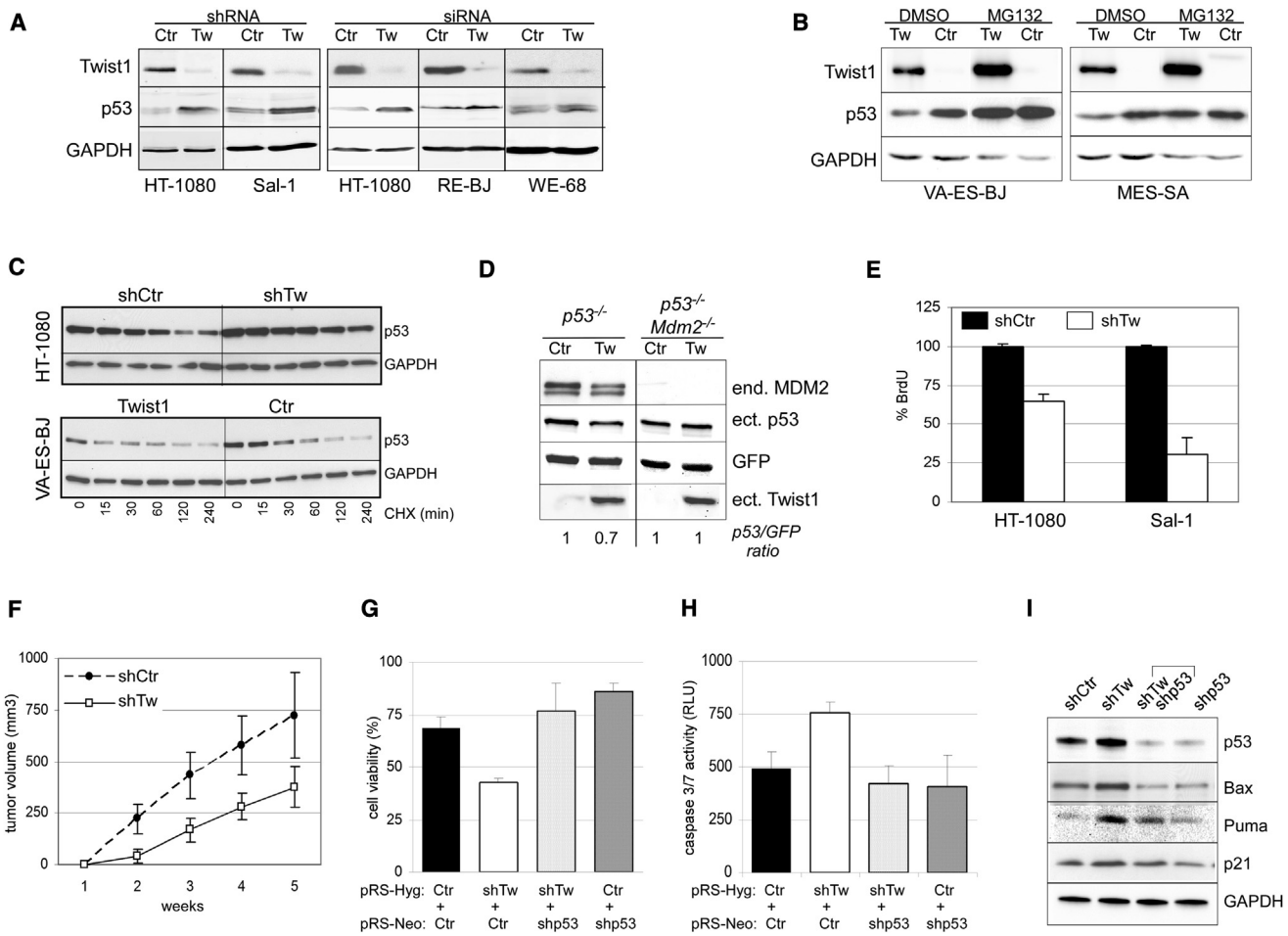


Figure 2. Modulation of Twist1 Expression Affects p53 Response and Tumorigenicity in Sarcoma Cells

(A) Immunoblot of cells silenced for endogenous Twist1 expression by either shRNA (left) or siRNA (right). As a control (Ctr), shLuc and nontargeting siRNA pool were used, respectively. Protein levels were normalized to GAPDH.

(B) Immunoblot of cells ectopically expressing either Twist1 (Tw) or GFP, as a control (Ctr). Cells were exposed to MG132 (10 μM) or vehicle (DMSO) for 4 hr, then harvested.

(C) Cycloheximide-chase assays (CHX, 100 μg/ml) in Twist1-silenced HT-1080 and in VA-ES-BJ engineered to express ectopic Twist1. shGFP (shCtr) and GFP (Ctr) were used as controls, respectively.

(D) Immunoblot of $p53^{-/-}$ and $p53^{-/-}$;$Mdm2^{-/-}$ MEF cotransfected with ectopic p53 (5 μg) and either myc-Twist1 (Tw) or empty control vector (Ctr) (15 μg). GFP (1 μg) was used as a control of transfection. p53 expression levels (ect. p53) were corrected for transfection efficiency (p53/GFP ratio). This result was confirmed on three independent experiments.

(E) BrdU incorporation assay in Twist1-silenced HT-1080 and Sal-1 cells. Histograms represent the mean plus SD.

(F) Xenograft growth of Twist1-silenced HT-1080 cells. Curves represent the average tumor volumes ± SD.

(G and H) HT-1080 cells were engineered to express combination of two shRNAs in pRS-Hyg and -Neo, as indicated. Controls (Ctr) were shGFP and shLuc. Cells were seeded overnight and then were shifted to serum-free medium. Cell viability (G) and caspase 3/7 activation (H) were assessed 48 hr after starvation. Histograms represent mean values plus SD.

(I) HT-1080 cells engineered and serum starved as in (G) and (H) were immunoblotted for the indicated proteins.

See also Figure S2.

Twist1 Enhances MDM2-Mediated Degradation of p53

We then assessed the effect of modulation of Twist expression in a series of human sarcoma cell lines expressing wild-type p53 (Figures S1A and S1B). Downregulation of the endogenous Twist1 by either stable expression of Twist1-specific shRNAs (Ansieau et al., 2008; Yang et al., 2004) or transient transfection of siRNAs stabilized p53 (Figure 2A). Conversely, ectopic Twist1 expression in $Twist1^{-}$ sarcoma cells reduced the p53 protein level, without affecting p53 transcription (data not shown). This downregulation was reversed by proteasome inhibition, suggesting that Twist1 promotes p53 proteasomal degradation (Figure 2B). Accordingly, cycloheximide (CHX)-chase assays indicated that the p53 half-life was increased from ~1 hr to ~3 hr in Twist1-silenced HT-1080 cells, whereas it was reduced from ~45 min to ~20 min in VA-ES-BJ expressing ectopic Twist1 (Figure 2C). Moreover, cotransfection of Twist1 together with p53 resulted in a slight but consistent reduction in p53 levels (~30%) in MDM2-proficient ($p53^{-/-}$) but not in MDM2-deficient

(*p53*$^{-/-}$; *Mdm2*$^{-/-}$) mouse embryo fibroblasts (MEF) null for endogenous p53 (Figure 2D), supporting a role for MDM2 in Twist1-induced downregulation of p53.

Silencing of Twist1 Activates a p53 Response in Sarcoma Cells

Under standard culture conditions, Twist1-depleted cells exhibited impaired cell growth compared to control cells, as revealed by longer doubling time (~1.3 and 1.9 times longer for HT-1080 and Sal-1, respectively; data not shown), decreased BrdU incorporation (Figure 2E), and reduced S-phase fraction (Figure S2A). Moreover, Twist1 knockdown was associated with induction of acidic β-galactosidase activity (SA-β-gal) (Figures S2B and S2C), suggesting spontaneous premature senescence, and with reduced tumorigenicity in xenograft models (Figures 2F and S2D). Twist1 depletion correlated also with enhanced sensitivity to serum starvation and UV radiation (Figures 2G, 2H, and S2C). This was paralleled by induction of p53 target genes (Figure 2I) and was rescued by silencing of p53 (Figures 2G–2I and S2E). No relevant perturbation in cell growth was observed following Twist1 silencing in sarcoma cells that were either homozygously deleted (SAOS-2) or mutated (SK-UT-1) for *TP53* or that overexpressed MDM2 (SJSA) (Figure S2A).

Twist1 antagonizes oncogene-induced apoptosis at least in part by interfering with the ARF/p53 pathway. However, a significant fraction of sarcomas and sarcoma-derived cell lines (including HT-1080, Sal-1, MES-SA, VA-ES-BJ, and WE68) are deficient for INK4a/ARF, and Twist1 can attenuate Myc-induced apoptosis in p53 wild-type/ARF null U2-OS cells (Figure S2F). This suggests that Twist1 impinges on p53 also independently of ARF.

Twist1-Mediated Antagonism of p53 Activity Does Not Require an Intact Basic Domain or Binding to p300/CBP

To shed light on the mechanisms of Twist-induced destabilization of p53, we characterized Twist1 domains required to antagonize the p53 response. Twist1 is known to regulate the transcription of several genes by binding their promoters through its basic DNA binding domain (Cakouros et al., 2010; Yang et al., 2010). We therefore investigated whether Twist1 modulated p53 response through a direct transcriptional mechanism. To this end, a series of Twist1 mutants defective for DNA binding (Spicer et al., 1996) (Figure 3A) were assayed for inhibition of p53-mediated transcription and apoptosis in E1A/Ras MEF (ER-MEF), which represent a well-defined setting of oncogene-induced/p53-dependent apoptosis (Lowe et al., 1993). In these cells, ectopic Twist expression attenuates p53-induced apoptosis (Maestro et al., 1999), whereas silencing of endogenous Twist1 results in enhanced stress sensitivity (Figures S3A–S3E). Despite loss of DNA binding activity, Twist1 basic domain mutants retained ability to protect ER-MEF from p53-mediated apoptosis (Figures 3B and S3F) and to repress a p53-responsive promoter (Figure S3G). More importantly, similar to full-length Twist1, the mutant carrying a deletion of the whole basic domain (Δb) was capable of converting nontumorigenic RE-BJ into tumorigenic cells (Figures 3C and S3H). Taken together, these data indicated that Twist1 could antagonize p53 and contribute to cancer development through an E-box-independent mechanism.

Twist1 has been hypothesized to interfere with p300/CBP-mediated activation of p53 (Ansieau et al., 2008; Hamamori et al., 1999; Shiota et al., 2008). To test this hypothesis, we generated a Twist1 mutant devoid of the major p300/CBP binding region (Δ30-60) (Figure 3A). The p300/CBP binding region overlaps Twist1 nuclear localization signal (NLS), and, therefore, the Δ30-60 mutant lost nuclear localization (Figure 3D). To circumvent this problem, an ectopic NLS (KRKK) was inserted at the N terminus (Δ30-60NLS). Despite impaired p300 binding, Twist1 Δ30-60NLS retained the power of repressing p53-mediated transcription (Figures S3G–S3I) and promoting cell survival (Figure 3D). This result demonstrates that the binding to p300/CBP is dispensable for Twist1-mediated inhibition of p53. Moreover, the fact that only the mutant expressed in the nucleus was capable of preventing p53-dependent apoptosis indicates that Twist1 requires nuclear localization to antagonize p53.

Twist and p53 Establish Tail-Tail Interaction that Involves the *Twist box* and p53 C-Terminal Regulatory Domain

It has been recently proposed that p53 may interact with the N terminus of Twist1 (Shiota et al., 2008). We then hypothesized that Twist1 could inhibit p53 response in sarcomas by directly targeting p53. Immunoprecipitation of endogenous Twist1 resulted in coprecipitation of endogenous p53 (Figure 4A), indicating that Twist1 does interact with p53 in sarcoma cells. The interaction is direct, as demonstrated by GST pull-down and coprecipitation assays using GST-Twist1 and His-p53 recombinant proteins (Figure 4B).

GST pull-down experiments confirmed the suggested weak interaction between p53 and the very N terminus of Twist1 (Figure S4A). However, we have shown that the deletion of this region does not affect the ability of Twist1 to antagonize p53-dependent transcription and apoptosis (Δ30-60NSL) (Figures 3D and S3G), ruling out a major biological relevance for this particular interaction. Instead, we found that p53 binds robustly to the C terminus of Twist1. In fact, both in vitro and in vivo (Figures S4A and S4B) coprecipitation experiments indicated that Twist1 engages a 20-aa stretch, corresponding to the highly conserved C-terminal region named *Twist box* (Bialek et al., 2004), to interact with p53 (Figure 4C).

The specificity of the binding was confirmed by GST pull-down using a *Twist box* peptide displayed from the active site loop of thioredoxin (T175-199), implying that this minimal region is both required and sufficient for p53 interaction. More importantly, *Twist box*-defective mutants were impaired in their ability to antagonize p53-depedent apoptosis (Figure 4D) and transcription (Figure S3G). This corroborates the relevance of the *Twist box* in the inhibition of p53. Intriguingly, this domain is conserved also in Twist2 and, in fact, Twist2 protected ER-MEF and bound p53 as efficiently as Twist1 (Figure 4D; Figure S4C).

Reciprocal mapping experiments revealed that p53 interacts with Twist through its C-terminal regulatory domain (aa 354–393) (p53CTD) (Figures 4E and S4D). These results were also corroborated by in silico prediction of protein:protein interaction. Docking simulation of human Twist1 and p53 models provided the best docking score for p53CTD and Twist1 *Twist box*, with

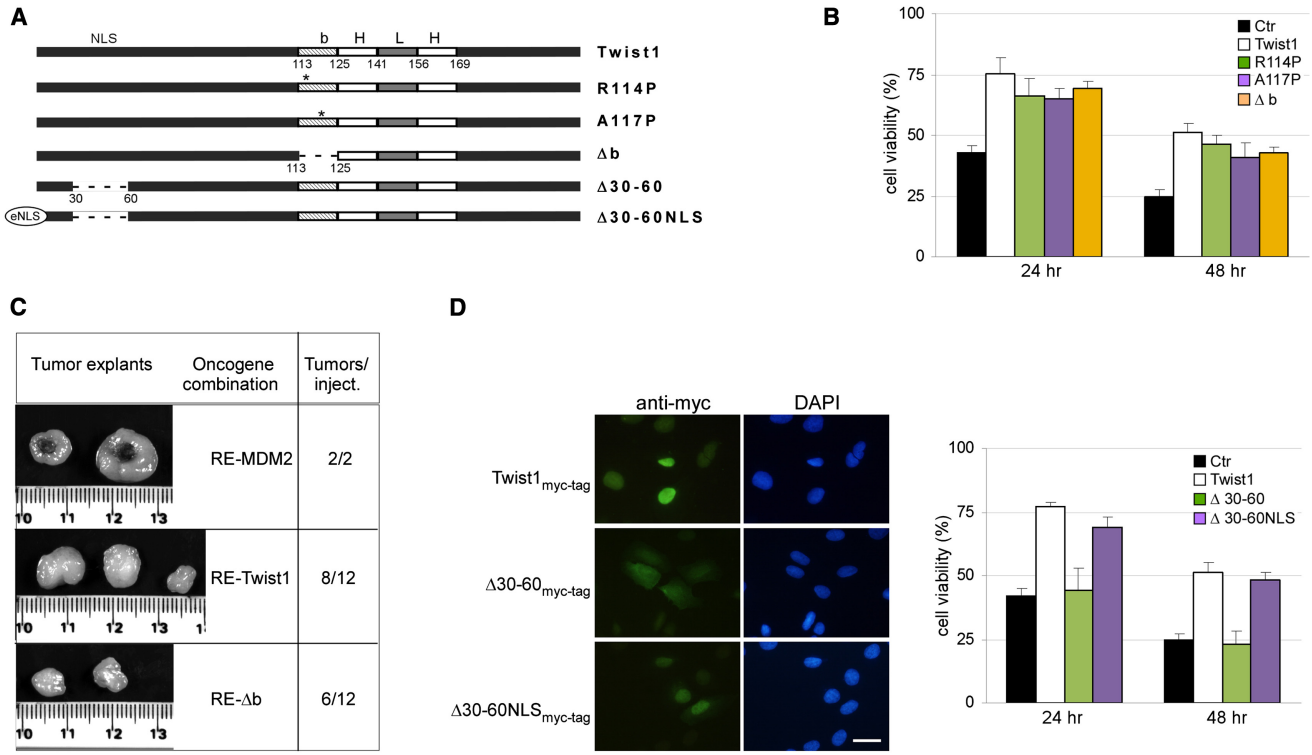


Figure 3. Twist1 Inhibits p53 also Independently of E-Box and p300/CBP Binding

(A) A schematic representation of the mouse Twist1 and various mutants. A114P and A117P carry a destabilizing proline into the basic domain (b); Δb is a whole basic-domain deletion mutant; NLS indicates Twist1 nuclear localization signal, bHLH the basic Helix-Loop-Helix domain; and eNLS denotes the ectopic nuclear localization signal.

(B) Cell viability of ER-MEF engineered as indicated after oncogene-mediated/p53-dependent apoptosis (0.1 μg/ml doxorubicin, 24 and 48 hr). Histograms indicate the mean percentage of treated/untreated cells, plus SD. Ctrl are control vector-infected cells.

(C) Representative tumor explants from RE-BJ cells engineered to express MDM2, Twist1, or Δb Twist1 (left). The number of tumors generated in a representative set of injections is reported on the right. Pictures were taken at week 5.

(D) Left: Immunofluorescence of ER-MEF engineered to express the indicated Twist1 myc-tag constructs. Nuclei are stained with DAPI. Scale bar: 50 μm. Right: Cell viability of ER-MEF engineered as indicated after apoptotic challenge (0.1 μg/ml doxorubicin, 24 and 48 hr). Histograms indicate the mean percentage of treated/untreated cells, plus SD.

See also Figure S3.

a predicted interface of the complex of 980 Å^2 and a highly significant lowest energy cluster population (187/500 individuals) (Figures 5A and 5B). In agreement with GST pull-down data, docking simulations ruled out a major role for other regions of p53 in the interaction with Twist1, including the p53 "core domain" (Cho et al., 1994) (lowest energy cluster: 55/500 individuals). The relevance of *Twist box* in Twist:p53 interaction was corroborated by the finding that single amino acid substitutions in the *Twist box* affected the binding in vitro to p53 (Figure 5C).

Twist1 Hinders p53 Phosphorylation at Ser392

Posttranslational modifications of C-terminal residues are involved in the regulation of p53 activity and stability (Xu, 2003). Intriguingly, in silico docking experiments indicated that one of the p53 residues involved in the interaction with Twist1 is the highly conserved Ser392 (Ser389 in mouse). In fact, in the Twist1:p53 complex, Ser392 is directly engaged in hydrogen bonds with Arg191 and Ala200 of Twist1 and is shielded by the Twist1 C terminus (Val189-His202) (Figures 5B and 5D).

Phosphorylation of Ser392 has been implicated in the regulation of p53 stability and activity (Cox and Meek, 2010; Hupp et al., 1992; Kapoor et al., 2000; Sakaguchi et al., 1997; Yap et al., 2004). Moreover, mice expressing a p53 mutant that cannot be phosphorylated at Ser389 (S389A) show impaired p53 response, with increased sensitivity to chemical and UV-induced carcinogenesis (Bruins et al., 2004; Hoogervorst et al., 2005).

We then hypothesized that Twist1 may affect p53 by directly inhibiting Ser392 phosphorylation. Indeed, we found that silencing of Twist1 was associated with a significant increase in the fraction of p53 phosphorylated at Ser392 (PSer392). Conversely, PSer392 was diminished in the cells engineered to express Twist1 or p53 binding-proficient Twist1 mutants but was unaffected in the cells expressing *Twist box*-deleted mutants (Figures 6A and S5A). Moreover, transient cotransfection experiments indicated that, under conditions where ectopic Twist1 efficiently promoted degradation and repressed transcriptional activity of wild-type p53, Twist1-mediated inhibition was impaired toward Ser392 mutants of p53 (Figures 6B

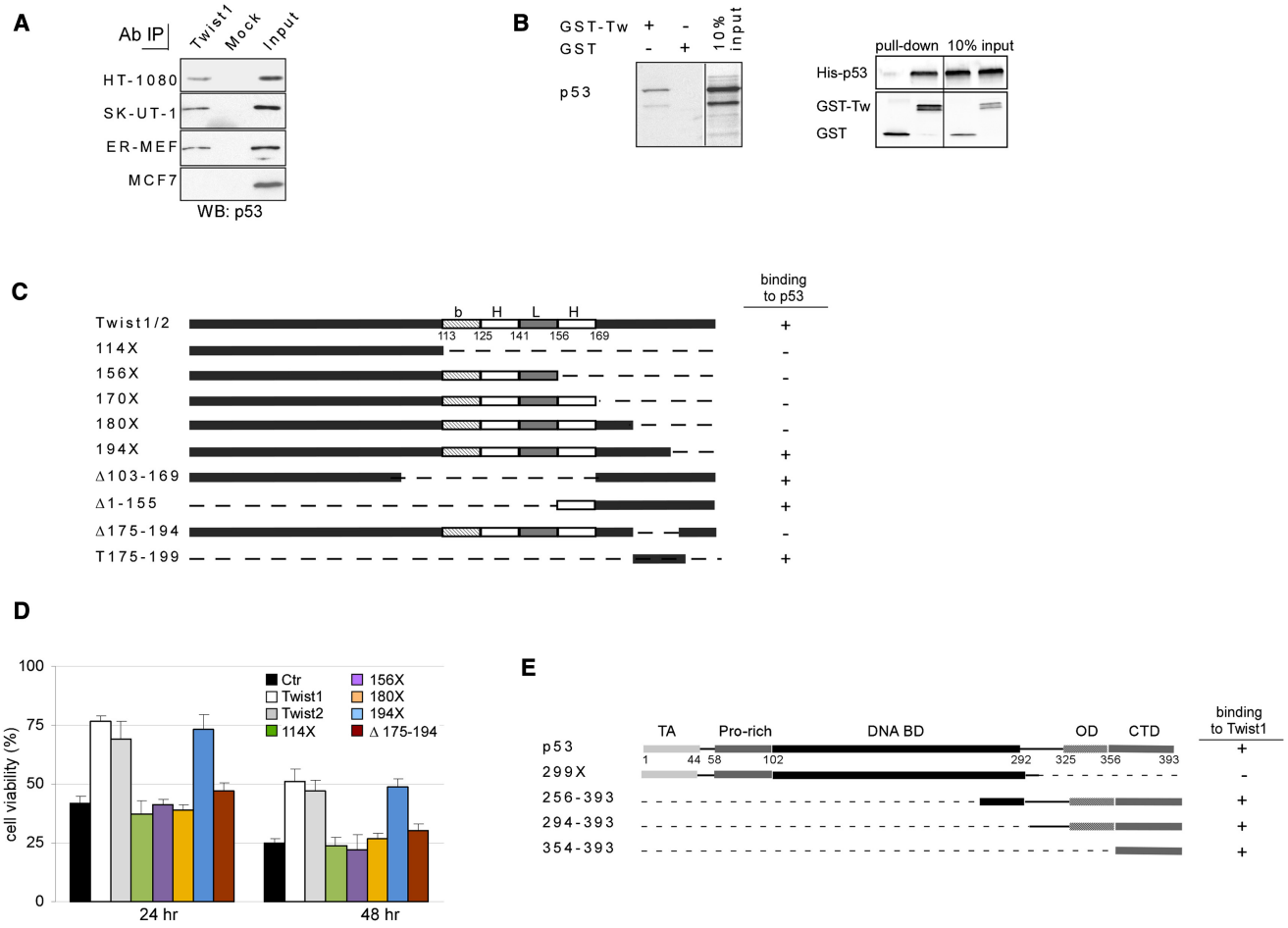


Figure 4. Twist1 and p53 Bind Directly through the C Termini

(A) Cells lysates from HT-1080, SK-UT-1, and ER-MEF were incubated with either Twist1 monoclonal antibody (Twist1) or nonimmune IgG (Mock), immunoprecipitated, and then blotted for coprecipitated p53 using anti-p53 antibodies. Twist1-negative MCF7 cells were used as a negative control. Input represents 10% of the whole cell lysate prior immunoprecipitation.

(B) Left panel: ^{35}S-labeled in vitro translated p53 (IVT-p53) was incubated with equivalent amounts of GST-Twist1 or GST, used as a negative control. Complexes were visualized by autoradiography. Right panel: recombinant His-p53 was incubated with equal amounts of glutathione agarose-bound GST or GST-Twist1. After SDS-PAGE, complexes were detected with anti-GST and anti-His antibodies.

(C) A schematic representation of the mouse Twist and various Twist1 mutants (left) and summary of their ability to interact with p53 based on GST pull-down experiments (right).

(D) Cell viability of ER-MEF engineered as indicated after apoptotic challenge (0.1 μg/ml doxorubicin, 24 hr and 48 hr). Histograms indicate the mean percentage viability plus SD.

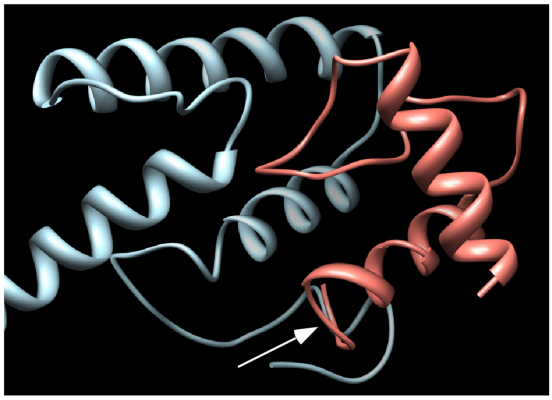
(E) A schematic representation of human p53 and derivative mutants (left) and summary of their ability to interact with Twist1 based on GST pull-down experiments (right).

See also Figure S4.

and S5B). Finally, although it is still unclear what kinase phosphorylates Ser392 in vivo, casein kinase 2 (CK2) has been shown to target Ser392 in vitro (Cox and Meek, 2010), and we found that in vitro CK2-mediated phosphorylation at Ser392 was attenuated in the presence of recombinant Twist1 (Figure S5C).

Twist1 failed to significantly affect the acetylation of Lys373 and Lys382 of p53, two residues involved in p53 stabilization and included in the region of p53 engaged in the interaction with Twist1 (Figures S5D–S5F). Although we cannot rule out that Twist1 may alter other posttranscriptional modifications relevant for p53 activity and stability, our data indicate that Twist1 attenuates the p53 response at least in part by impinging on Ser392 phosphorylation. Intriguingly, different from Lys373 and Lys382 acetylation, Ser392 phosphorylation is triggered in response to oncogene activation, both in human (Figures S5D–S5F) and mouse fibroblasts (Figure S5G), and this correlates with p53 induction. In the same cells, oncogene-induced transformation was associated also with increased Twist1 levels (Figures S5G and S5H). This suggests that the activation of Twist1 may represent a mechanism elicited by oncogene-challenged cells to quench p53 response during transformation.

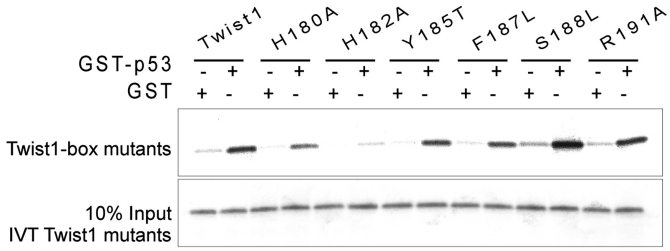
A



B

Human Twist1 residue and atom	Human p53 residue and atom	Distance (Å)
Ile134 O	Gly360 O	2.9
Tyr155 OH	Glu358 N	3.2
	Glu358 O	2.7
	Gly356 O	3.0
His180 NE2	Gly361 N	3.2
Arg182 NH1	Asp391 OD1	2.7
	Glu388 OE1	2.8
Arg182 NH2	Asp393 OD2	2.8
Arg191 NH1	Ser392 OG	2.8
	Asp391 OD1	2.8
	Ser392 N	3.1
Ala200 O	Ser392 N	2.9
His202 N	Asp391 OD2	3.2

C



D

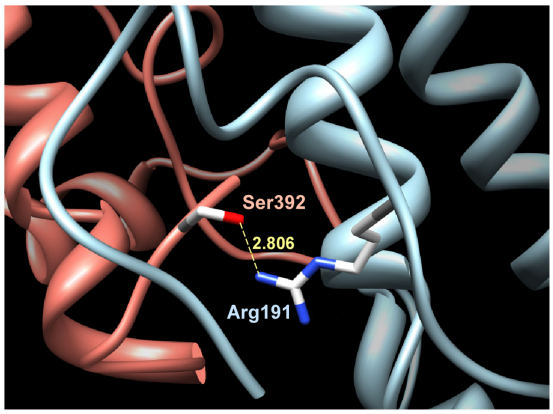


Figure 5. The *Twist box* Is Necessary for the Interaction with p53

(A) Ribbon representation of the interaction between p53 (red) and Twist1 (blue). The arrow indicates the *Twist box*:p53 interface.

(B) A summary of the residues of human Twist1 and human p53 involved in their binding, the functional groups of these residues involved in the interaction, and the distance (in Å) between the corresponding interacting functional groups as assessed by docking simulation. In mouse Twist1, the region involved in the binding spans Ile138-His206.

(C) GST or GST-p53 pull-downs of IVT-*Twist box* or IVT-*Twist box* carrying the indicated amino acid substitutions.

(D) Magnification of the ribbon representation of *Twist box* (blue):p53 CTD (red) interaction. The hydrogen bond formed between p53 Ser392 and Twist1 Arg191 and the distance between these two residues in Å are indicated in yellow.

Twist1 Facilitates p53 Degradation by Increasing the Affinity of p53 for MDM2

Modifications of p53CTD are known to modulate its susceptibility to MDM2-mediated degradation. We therefore sought to investigate the role of Ser392 in p53 degradation. CHX-chase experiments in SAOS-2 and *p53*$^{-/-}$ HCT116 cells engineered to stably express p53 mutants at codon 392 indicated that p53-S392E, a mutant mimicking constitutive phosphorylation at Ser392, displayed a longer half-life than did the p53-S392A phosphorylation-deficient mutant (Figure 6C). On a side note, we noticed that both Ser392 mutants were more tolerated compared to p53 WT, suggesting that Ser392 status affects both p53 turnover and tumor-suppressive activity. Moreover, the differential stability of Ser392 mutants was easier to appreciate if the constructs were expressed following stable retroviral infection instead of acute transfection, likely because of transfection-induced stress response.

Intriguingly, the amount of MDM2 coimmunoprecipitated with p53-S392E was significantly reduced (~50%) compared to the amount bound to the wild-type p53 and p53-S392A (Figure 6D). This suggests that phosphorylation of Ser392 makes p53 less prone to binding to MDM2, and hence to MDM2-driven degradation. In this scenario, by preventing Ser392 phosphorylation, Twist1 might affect p53:MDM2 association. In agreement with this hypothesis, we found that the increase in the overall levels of p53 detected in HT-1080 cells following Twist1 knockdown correlated with a slight but consistent decrease (~30%) in the amount of p53 complexed to MDM2 (Figures 6E and S5I).

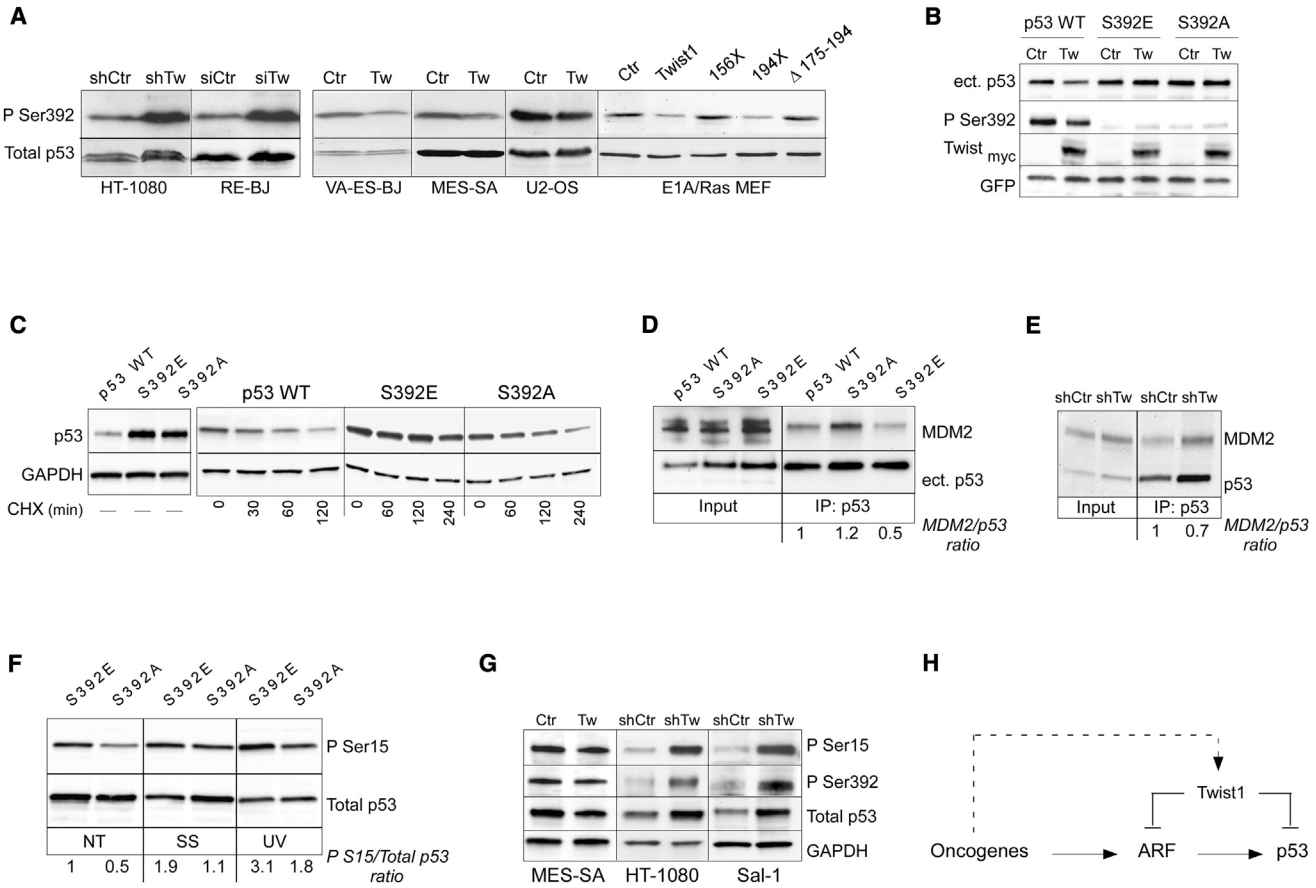


Figure 6. Twist1 Hinders Phosphorylation of p53 at Ser392 and Affects p53:MDM2 Interaction

(A) Immunoblot for p53 phosphorylated at Ser392 (P Ser392) of sarcoma cells silenced for Twist1 expression (left) or engineered to express ectopic Twist1 or Twist1 mutants (right). Because modulation of Twist1 expression affects p53 expression levels, to better appreciate the effect of Twist1 on Ser392 phosphorylation, samples were unevenly loaded to tentatively equalize the signals for total p53.

(B) Immunoblot of Twist1-negative *p53*$^{-/-}$ HCT116 transfected with wild-type p53 or p53 mutants (4 μg) as indicated together with either myc-Twist1 (Tw) or empty control vector (Ctr) (20 μg). GFP (1 μg) was included for normalization of transfection efficiency. This result was confirmed on three independent experiments.

(C) Cycloheximide-chase assays (CHX, 250 μg/ml) in SAOS-2 cells infected with retroviral vectors encoding p53 WT, p53 S392E (phospho-mimic), or p53 S392A (phospho-impaired).

(D) SAOS-2 engineered to express p53 WT, S392A, or S392E p53 were treated with MG132 (10 μM, 8 hr), then were immunoprecipitated for p53. Immunoblots were probed with anti-MDM2 and -p53 antibodies, and the amount of MDM2 coprecipitated was calculated as MDM2/p53 ratio. This result was confirmed in two independent experiments.

(E) Twist1-silenced (shTw) and control (shCtr) HT-1080 were treated with MG132 (10 μM, 8 hr), immunoprecipitated for endogenous p53, and then probed for MDM2 and p53. The amount of MDM2 bound to p53 was calculated as MDM2/p53 ratio. This experiment was done three times with two different p53 antibodies (DO-1 and CM5).

(F) Immunoblot of *p53*$^{-/-}$ HCT116 engineered to express either S392A or S392E p53, under standard conditions (NT), UV radiation (20 J/m^2) or serum starvation (30 hr).

(G) Immunoblots for p53 phosphorylation at Ser15 and Ser392 in sarcoma cells after modulation of Twist1 expression. GAPDH was used for normalization.

(H) Schematic representation of the proposed mechanisms of Twist1 contribution to oncogene-induced transformation and attenuation of p53 response.

See also Figure S5.

Thus, Twist1 seems to promote p53 degradation by maintaining p53 in an MDM2-accessible state.

It has been recently shown that, besides the canonical N-terminal region (Kussie et al., 1996; Lin et al., 1994), p53 binds MDM2 also with the CTD, and this interaction is hampered by p53CTD modifications (Poyurovsky et al., 2010). Thus, Twist1 could interfere with this alternative mechanism of MDM2-mediated degradation of p53. However, an interplay between Ser392 and Ser15 of p53 has been proposed (Kapoor et al., 2000), and phosphorylation at Ser15 is well known to inhibit MDM2 from binding N terminus p53 (Shieh et al., 1997). Therefore, we investigated a possible cross-talk between these two serines. We found that p53-S392E displayed a greater extent of phosphorylation at Ser15 than did p53-S392A (Figure 6F). Instead, constitutive phosphorylation at Ser15 (S15E) failed to affect the extent of phosphorylation at Ser392 (Figure S5J). This suggests a directional control of Ser15 phosphorylation by Ser392 status. Moreover, modulation of Twist1 expression in

sarcoma cells correlated with concordant variations in Ser392 and Ser15 phosphorylation (Figure 6G). Thus, besides interfering with the p53CTD:MDM2 interaction, Twist1 might indirectly affect also the canonical route of MDM2-mediated p53 degradation, suggesting the existence of multiple levels of controls of Twist1 over p53 (Figure 6H).

DISCUSSION

By investigating the role of Twist1 in the context of sarcomas, this study highlights an alternative mechanism of p53 inactivation and provides evidence that Twist1 promotes MDM2-mediated degradation of p53 by directly interacting with its C-terminal regulatory domain and by interfering with key phosphorylations.

We found that although Twist1 expression pattern in carcinomas is compatible with its proposed role in malignant progression, the diffuse and strong Twist1 accumulation observed in sarcomas suggests that Twist1 activation is an intrinsic component of the transformed phenotype of tumor cells of mesenchymal origin. This hypothesis is supported by the finding that, in these tumors, Twist1 accumulation often associates with *TWIST1* copy-number gain, a result that is in line with recent evidence that one of the most frequent copy-number variations in sarcomas involves the *TWIST1* locus (Menghi-Sartorio et al., 2001; Taylor et al., 2008).

In addition, we found that, in LS, Twist1 overexpression is mutually exclusive with MDM2 positivity. In LMS, Twist1 accumulation clustered among p53 wild-type tumors, although the correlation was not statistically significant. This may be in part due to the small sample size of these clinically rare tumors. On the other hand, although early studies suggested that *TP53* mutation and MDM2 overexpression were mutually exclusive, subsequent studies have demonstrated that this is not a general rule. Indeed, overexpression/amplification of MDM2 and *TP53* mutation can coexist in the same tumor, likely because both p53 and MDM2 are provided with functions independent of each other (Cordon-Cardo et al., 1994; Ito et al., 2011; Manfredi, 2010). Similarly, Twist1 is also provided with p53-independent functions that may play a role in sarcomagenesis (e.g., inhibition of differentiation) and might account for Twist1 and MDM2 coexpression observed in six LMS retaining wild-type *TP53.*

Most evidence linking Twist1 to cancer refers to the ability of Twist proteins to modulate the expression of target genes, including ARF, thus affecting p53. This study provides evidence that Twist1 affects p53 also through an E-box-independent mechanism. In fact, we found that Twist1 mutants defective for DNA binding retain the ability to interfere with the p53 response. This indicates that Twist1 may participate to tumorigenesis and promote the bypass of p53 failsafe programs apparently through molecular mechanisms different from those that facilitate EMT in carcinomas.

Intriguingly, we found that Twist1 establishes a direct interaction with p53 through the *Twist box* and that this interaction is critical for Twist1 inhibition of p53. The *Twist box* corresponds to a highly conserved 20-aa stretch at the C terminus of the protein. Through the *Twist box,* Twist binds to and inhibits RunX2 (Bialek et al., 2004), MEF2 (Spicer et al., 1996), ATF4 (Danciu et al., 2012), and RelA (Šošić et al., 2003). This suggests that the binding via *Twist box* is a leitmotif in the inhibition exerted by Twist on other transcription factors. Here, we demonstrate that Twist employs the *Twist box* to interact with the CTD regulatory domain of p53. By binding p53CTD, Twist1 hinders Ser392 phosphorylation, and this correlates with increased p53 sensitivity to degradation. Although Twist1 failed to significantly affect Lys373 and Lys382 acetylation, we cannot exclude that, by interacting with p53CTD, Twist1 may influence other modifications that affect p53 functions. Nevertheless, the fact that Ser392 is phosphorylated in response to oncogene activation and modulates p53 activity and stability corroborates the concept that Twist1 participates to sarcomagenesis at least in part by interfering with Ser392-mediated p53 activation.

How may *Twist box*:p53CTD interaction affect p53 response? Recently, Prives's group has demonstrated that, besides the canonical N terminus, p53 engages also the CTD region to bind MDM2, and this interaction is hampered by p53CTD posttranslational modifications (Poyurovsky et al., 2010). Although the authors addressed the effect of acetylation on p53CTD:MDM2 complex formation, they argued that other p53CTD modifications are likely to impinge on this interaction. By using Ser392 mutants, we collected data suggesting that Ser392 phosphorylation reduces p53 affinity to MDM2. Thus, by hindering Ser392 phosphorylation, Twist1 might favor p53CTD:MDM2 complex formation and hence p53 degradation. Yet we found that Ser392 status influences also the extent of phosphorylation at Ser15 that inhibits the interaction between N terminus p53 and MDM2. Then, by acting on Ser392, Twist1 might in turn impinge on Ser15, thus facilitating also the canonical route of MDM2-mediated degradation of p53.

Finally, in agreement with recent observations (Kogan-Sakin et al., 2011; Lee and Bar-Sagi, 2010), we found that oncogene-challenged human and mouse fibroblasts display increased levels of Twist1. This suggests a scenario where oncogenic transformation results in the induction of Twist1. In turn, Twist1 promotes the bypass of oncogene-induced p53 safeguard responses by both inhibiting the ARF/p53 signaling and by directly interacting with p53 and facilitating its degradation (Figure 6H).

In conclusion, this study sheds light on the mechanisms of Twist1-mediated inactivation of p53 and adds another important piece of information on the role of p53CTD in the regulation of p53 tumor-suppressive activity. Although focused on sarcomas, our study proposes a model that might also apply to other tumor histotypes and provides the proof of principle that targeting Twist1:p53 interaction may offer additional avenues for the treatment of tumors.

EXPERIMENTAL PROCEDURES

Immunohistochemistry and FISH

Primary sarcoma samples and corresponding normal tissues were retrieved from the Treviso General Hospital and the San Raffaele Institute tissue banks, where they were analyzed with patients' consent. Specimens were deidentified before analysis, and the study was approved by the IRB of CRO, Treviso General Hospital, and San Raffaele Institute. No patient had received radio- or chemotherapy prior to surgery. Immunohistochemistry was performed on 5-μm sections with the following antibodies: Twist1 (Twist2C1a, Santa Cruz), Twist 2 (ab6603, Abcam), p53 (DO-7, Labvision), and MDM2 (1F2, Oncogene Science). FISH was performed following standard protocols on 19 cases for which frozen material was available.

TP53 Mutation Analysis

Mutation analysis of *TP53* was performed by PCR direct-sequencing on genomic DNA extracted from paraffin-embedded tissue sections, as previously described (Dei Tos et al., 1997).

Cells, Constructs, and Protein Expression Analyses

Transfections, retroviral infections, and reporter assays were performed as previously described (Demontis et al., 2006). Acute silencing of Twist1 was achieved with ON-TARGETplus SMART pool siRNAs (Dharmacon), according to the manufacturer's instructions. Stable silencing of Twist1 was achieved by retroviral infection of previously published Twist1-specific shRNAs (Ansieau et al., 2008; Yang et al., 2004). shGFP and shLuciferase (shLuc) were used as negative controls. Protein lysates were separated by SDS-PAGE and were transferred onto nitrocellulose membrane (Protran, Whatman). Expression analyses were performed with Odyssey Infrared (Li-Cor Biosciences) and Chemidoc (BioRad) Imaging systems, using the antibodies indicated in Supplemental Experimental Procedures. Immunofluorescence and SA-β-gal staining were performed as previously described (Di Micco et al., 2006; Seger et al., 2002). GST pull-downs and in vitro CK2 assay were performed as described in Supplemental Experimental Procedures.

Apoptosis, BrdU Incorporation, and FACS Analyses

Cell viability was assessed by Trypan blue and Viacount assay (Guava PCA) and was calculated as mean percentage of cells that survived the apoptotic stress (100× viable treated cells/untreated cells). Experiments were done in triplicate on at least two different retroviral infections. Caspase 3/7 activation and BrdU incorporation were determined with the Apo-ONE Caspase-3/7 and the BrdU Kits (Promega). FACS analyses were done with the Cytomics FC 500 (Beckman Coulter).

In Vivo Tumorigenicity Assay

Experiments on animals were performed in accordance with national regulations and approved by the CRO animal ethics committee. RE-BJ cells (5×10^6) stably expressing MDM2, dnp53, shp53, or Twist were subcutaneously injected into each flank of six-week-old athymic nude mice (Hsd:Athymic nude-nu, Harlan) as previously described (Di Micco et al., 2006; Seger et al., 2002) For pRetroSuper-shLuc and pRetroSuper-shTwist HT-1080, 1×10^6 cells were used. Tumor size was monitored weekly. Mice were sacrificed at week 5. Tumor volume was calculated as $2r^3$.

Protein Modeling and Docking Simulations

Three-dimensional structure models of Twist1 and p53 were built using the web server SAM-T08 (Karplus, 2009) and further minimized by simulated annealing using the program CNS (Brünger et al., 1998). Docking calculations were carried out by the web server ClusPro 2.0 (Comeau et al., 2007).

Statistical Analysis

Data shown are means ± SD of at least three independent experiments. Comparisons of proportions were performed using two-tailed Fisher's exact test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.08.003.

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REFERENCES

Ansieau, S., Bastid, J., Doreau, A., Morel, A.P., Bouchet, B.P., Thomas, C., Fauvet, F., Puisieux, I., Doglioni, C., Piccinin, S., et al. (2008). Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell *14*, 79–89.

Barnes, R.M., and Firulli, A.B. (2009). A twist of insight—the role of Twist-family bHLH factors in development. Int. J. Dev. Biol. *53*, 909–924.

Bialek, P., Kern, B., Yang, X., Schrock, M., Sosic, D., Hong, N., Wu, H., Yu, K., Ornitz, D.M., Olson, E.N., et al. (2004). A twist code determines the onset of osteoblast differentiation. Dev. Cell *6*, 423–435.

Borden, E.C., Baker, L.H., Bell, R.S., Bramwell, V., Demetri, G.D., Eisenberg, B.L., Fletcher, C.D., Fletcher, J.A., Ladanyi, M., Meltzer, P., et al. (2003). Soft tissue sarcomas of adults: state of the translational science. Clin. Cancer Res. *9*, 1941–1956.

Bruins, W., Zwart, E., Attardi, L.D., Iwakuma, T., Hoogervorst, E.M., Beems, R.B., Miranda, B., van Oostrom, C.T., van den Berg, J., van den Aardweg, G.J., et al. (2004). Increased sensitivity to UV radiation in mice with a p53 point mutation at Ser389. Mol. Cell. Biol. *24*, 8884–8894.

Brünger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. (1998). Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. *54*, 905–921.

Cakouros, D., Raices, R.M., Gronthos, S., and Glackin, C.A. (2010). Twist-ing cell fate: mechanistic insights into the role of twist in lineage specification/differentiation and tumorigenesis. J. Cell. Biochem. *110*, 1288–1298.

Castanon, I., and Baylies, M.K. (2002). A Twist in fate: evolutionary comparison of Twist structure and function. Gene *287*, 11–22.

Cho, Y., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346–355.

Coindre, J.M., Pédeutour, F., and Aurias, A. (2010). Well-differentiated and dedifferentiated liposarcomas. Virchows Arch. *456*, 167–179.

Comeau, S.R., Kozakov, D., Brenke, R., Shen, Y., Beglov, D., and Vajda, S. (2007). ClusPro: performance in CAPRI rounds 6-11 and the new server. Proteins *69*, 781–785.

Cordon-Cardo, C., Latres, E., Drobnjak, M., Oliva, M.R., Pollack, D., Woodruff, J.M., Marechal, V., Chen, J., Brennan, M.F., and Levine, A.J. (1994). Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res. *54*, 794–799.

Cox, M.L., and Meek, D.W. (2010). Phosphorylation of serine 392 in p53 is a common and integral event during p53 induction by diverse stimuli. Cell. Signal. *22*, 564–571.

Danciu, T.E., Li, Y., Koh, A., Xiao, G., McCauley, L.K., and Franceschi, R.T. (2012). The basic helix loop helix transcription factor Twist1 is a novel regulator of ATF4 in osteoblasts. J. Cell. Biochem. *113*, 70–79.

Dei Tos, A.P., Maestro, R., Doglioni, C., Piccinin, S., Libera, D.D., Boiocchi, M., and Fletcher, C.D. (1996). Tumor suppressor genes and related molecules in leiomyosarcoma. Am. J. Pathol. *148*, 1037–1045.

Dei Tos, A.P., Doglioni, C., Piccinin, S., Maestro, R., Mentzel, T., Barbareschi, M., Boiocchi, M., and Fletcher, C.D. (1997). Molecular abnormalities of the p53 pathway in dedifferentiated liposarcoma. J. Pathol. *181*, 8–13.

Demontis, S., Rigo, C., Piccinin, S., Mizzau, M., Sonego, M., Fabris, M., Brancolini, C., and Maestro, R. (2006). Twist is substrate for caspase cleavage and proteasome-mediated degradation. Cell Death Differ. *13*, 335–345.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre', M., Nuciforo, P.G., Bensimon, A., et al. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638–642.

Feng, M.Y., Wang, K., Song, H.T., Yu, H.W., Qin, Y., Shi, Q.T., and Geng, J.S. (2009). Metastasis-induction and apoptosis-protection by TWIST in gastric cancer cells. Clin. Exp. Metastasis *26*, 1013–1023.

Fletcher, C.D.M., Unni, K.K., and Mertens, F., eds. (2002). World Health Organization Classification of Tumors: Pathology and Genetics of Tumors of Soft Tissue and Bone (Lyon, France: IARC Press).

Hall, K.L., Teneriello, M.G., Taylor, R.R., Lemon, S., Ebina, M., Linnoila, R.I., Norris, J.H., Park, R.C., and Birrer, M.J. (1997). Analysis of Ki-ras, p53, and MDM2 genes in uterine leiomyomas and leiomyosarcomas. Gynecol. Oncol. *65*, 330–335.

Hamamori, Y., Sartorelli, V., Ogryzko, V., Puri, P.L., Wu, H.Y., Wang, J.Y., Nakatani, Y., and Kedes, L. (1999). Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. Cell *96*, 405–413.

Helman, L.J., and Meltzer, P. (2003). Mechanisms of sarcoma development. Nat. Rev. Cancer *3*, 685–694.

Hoogervorst, E.M., Bruins, W., Zwart, E., van Oostrom, C.T., van den Aardweg, G.J., Beems, R.B., van den Berg, J., Jacks, T., van Steeg, H., and de Vries, A. (2005). Lack of p53 Ser389 phosphorylation predisposes mice to develop 2-acetylaminofluorene-induced bladder tumors but not ionizing radiation-induced lymphomas. Cancer Res. *65*, 3610–3616.

Hupp, T.R., Meek, D.W., Midgley, C.A., and Lane, D.P. (1992). Regulation of the specific DNA binding function of p53. Cell *71*, 875–886.

Isenmann, S., Arthur, A., Zannettino, A.C., Turner, J.L., Shi, S., Glackin, C.A., and Gronthos, S. (2009). TWIST family of basic helix-loop-helix transcription factors mediate human mesenchymal stem cell growth and commitment. Stem Cells *27*, 2457–2468.

Ito, M., Barys, L., O'Reilly, T., Young, S., Gorbatcheva, B., Monahan, J., Zumstein-Mecker, S., Choong, P.F., Dickinson, I., Crowe, P., et al. (2011). Comprehensive mapping of p53 pathway alterations reveals an apparent role for both SNP309 and MDM2 amplification in sarcomagenesis. Clin. Cancer Res. *17*, 416–426.

Kapoor, M., Hamm, R., Yan, W., Taya, Y., and Lozano, G. (2000). Cooperative phosphorylation at multiple sites is required to activate p53 in response to UV radiation. Oncogene *19*, 358–364.

Karplus, K. (2009). SAM-T08, HMM-based protein structure prediction. Nucleic Acids Res. *37* (Web Server issue), W492–W97.

Karreth, F., and Tuveson, D.A. (2004). Twist induces an epithelial-mesenchymal transition to facilitate tumor metastasis. Cancer Biol. Ther. *3*, 1058–1059.

Kogan-Sakin, I., Tabach, Y., Buganim, Y., Molchadsky, A., Solomon, H., Madar, S., Kamer, I., Stambolsky, P., Shelly, A., Goldfinger, N., et al. (2011). Mutant p53(R175H) upregulates Twist1 expression and promotes epithelial-mesenchymal transition in immortalized prostate cells. Cell Death Differ. *18*, 271–281.

Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J., and Pavletich, N.P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science *274*, 948–953.

Kwok, W.K., Ling, M.T., Yuen, H.F., Wong, Y.C., and Wang, X. (2007). Role of p14ARF in TWIST-mediated senescence in prostate epithelial cells. Carcinogenesis *28*, 2467–2475.

Lee, K.E., and Bar-Sagi, D. (2010). Oncogenic KRas suppresses inflammation-associated senescence of pancreatic ductal cells. Cancer Cell *18*, 448–458.

Li, Q.Q., Xu, J.D., Wang, W.J., Cao, X.X., Chen, Q., Tang, F., Chen, Z.Q., Liu, X.P., and Xu, Z.D. (2009). Twist1-mediated adriamycin-induced epithelial-mesenchymal transition relates to multidrug resistance and invasive potential in breast cancer cells. Clin. Cancer Res. *15*, 2657–2665.

Lin, J., Chen, J., Elenbaas, B., and Levine, A.J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev. *8*, 1235–1246.

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957–967.

Maestro, R., Dei Tos, A.P., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D.H., and Hannon, G.J. (1999). Twist is a potential oncogene that inhibits apoptosis. Genes Dev. *13*, 2207–2217.

Manfredi, J.J. (2010). The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes Dev. *24*, 1580–1589.

Menghi-Sartorio, S., Mandahl, N., Mertens, F., Picci, P., and Knuutila, S. (2001). DNA copy number amplifications in sarcomas with homogeneously staining regions and double minutes. Cytometry *46*, 79–84.

Mentzel, T., and Fletcher, C.D. (1995). Lipomatous tumours of soft tissues: an update. Virchows Arch. *427*, 353–363.

Poyurovsky, M.V., Katz, C., Laptenko, O., Beckerman, R., Lokshin, M., Ahn, J., Byeon, I.J., Gabizon, R., Mattia, M., Zupnick, A., et al. (2010). The C terminus of p53 binds the N-terminal domain of MDM2. Nat. Struct. Mol. Biol. *17*, 982–989.

Qin, Q., Xu, Y., He, T., Qin, C., and Xu, J. (2012). Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. Cell Res. *22*, 90–106.

Sakaguchi, K., Sakamoto, H., Lewis, M.S., Anderson, C.W., Erickson, J.W., Appella, E., and Xie, D. (1997). Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53. Biochemistry *36*, 10117–10124.

Seger, Y.R., García-Cao, M., Piccinin, S., Cunsolo, C.L., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. Cancer Cell *2*, 401–413.

Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325–334.

Shiota, M., Izumi, H., Onitsuka, T., Miyamoto, N., Kashiwagi, E., Kidani, A., Hirano, G., Takahashi, M., Naito, S., and Kohno, K. (2008). Twist and p53 reciprocally regulate target genes via direct interaction. Oncogene *27*, 5543–5553.

Šošić, D., Richardson, J.A., Yu, K., Ornitz, D.M., and Olson, E.N. (2003). Twist regulates cytokine gene expression through a negative feedback loop that represses NF-kappaB activity. Cell *112*, 169–180.

Spicer, D.B., Rhee, J., Cheung, W.L., and Lassar, A.B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science *272*, 1476–1480.

Stasinopoulos, I.A., Mironchik, Y., Raman, A., Wildes, F., Winnard, P., Jr., and Raman, V. (2005). HOXA5-twist interaction alters p53 homeostasis in breast cancer cells. J. Biol. Chem. *280*, 2294–2299.

Taylor, B.S., Barretina, J., Socci, N.D., Decarolis, P., Ladanyi, M., Meyerson, M., Singer, S., and Sander, C. (2008). Functional copy-number alterations in cancer. PLoS ONE *3*, e3179.

Tukel, T., Šošić, D., Al-Gazali, L.I., Erazo, M., Casasnovas, J., Franco, H.L., Richardson, J.A., Olson, E.N., Cadilla, C.L., and Desnick, R.J. (2010). Homozygous nonsense mutations in TWIST2 cause Setleis syndrome. Am. J. Hum. Genet. *87*, 289–296.

Valsesia-Wittmann, S., Magdeleine, M., Dupasquier, S., Garin, E., Jallas, A.C., Combaret, V., Krause, A., Leissner, P., and Puisieux, A. (2004). Oncogenic cooperation between H-Twist and N-Myc overrides failsafe programs in cancer cells. Cancer Cell *6*, 625–630.

Vesuna, F., Lisok, A., Kimble, B., and Raman, V. (2009). Twist modulates breast cancer stem cells by transcriptional regulation of CD24 expression. Neoplasia *11*, 1318–1328.

Vichalkovski, A., Gresko, E., Hess, D., Restuccia, D.F., and Hemmings, B.A. (2010). PKB/AKT phosphorylation of the transcription factor Twist-1 at Ser42 inhibits p53 activity in response to DNA damage. Oncogene *29*, 3554–3565.

Xu, Y. (2003). Regulation of p53 responses by post-translational modifications. Cell Death Differ. *10*, 400–403.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell *117*, 927–939.

Yang, M.H., Hsu, D.S., Wang, H.W., Wang, H.J., Lan, H.Y., Yang, W.H., Huang, C.H., Kao, S.Y., Tzeng, C.H., Tai, S.K., et al. (2010). Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat. Cell Biol. *12*, 982–992.

Yap, D.B., Hsieh, J.K., Zhong, S., Heath, V., Gusterson, B., Crook, T., and Lu, X. (2004). Ser392 phosphorylation regulates the oncogenic function of mutant p53. Cancer Res. *64*, 4749–4754.